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Effect of Concentration on the Microbiological Hydroxylation of Progesterone[1]

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The microbiological oxidation of progesterone to give 11α-hydroxyprogesterone is one of the most important routes to cortisone (Fieser and Fieser, 1959). Although a number of organisms can be used for this purpose (Eppstein *et al.*, 1956), the two most widely used are *Rhizopus nigricans* (Peterson *et al.*, 1952) and *Aspergillus ochraceus* (Dulaney *et al.*, 1955). It is of obvious importance and utility to conduct these hydroxylations using the highest possible concentrations of substrate. However, the published literature to date shows that rather low concentrations of progesterone were used. Thus, Peterson *et al.* (1952) recommend 0.5 to 1.0 g progesterone per L. Karow and Petsiavas (1956), working with *A. ochraceus* and using propylene glycol as a solvent for progesterone were able to achieve concentrations of 2.0 g per L in batch runs and 4.0 g per L in semicontinuous procedures, one of the chief limiting factors apparently being the toxicity of propylene glycol. The present communication reports the results of experiments which permit excellent 11α-hydroxylation of progesterone in concentrations as high as 20 to 50 g per L.

Materials and Methods

The cultures used in this study were *Rhizopus nigricans* Ehrb strain ATCC 6227b and *Aspergillus ochraceus* from the Merck Culture Collection.[3] Both organisms were brought into a vigorous, active stock culture from agar slants by multiple transfers in 16-ounce French square bottles using 100 ml of a 5 per cent malt medium under aerobic conditions (Weaver *et al.*, 1953). The bottles contained a ½-inch layer of glass beads which permitted uniform break-up of the surface growth for subsequent transfers. A culture was considered suitable for effecting a steroid transformation if it produced vigorous sporulation in 4 days under the above conditions. Vigorous vegetative growth of the organisms was achieved by use of the widely used Edamin,[4] glucose, corn steep liquor medium recommended by Peterson *et al.* (1952). A Fernbach flask containing 300 ml of this nutrient medium was autoclaved at 15 lb pressure for 15 min. On cooling it was inoculated with 2 per cent (6.0 ml) of the stock culture preparation. The flask was shaken at 28 C for 18 to 24 hr on a reciprocal shaker set to give 90 cpm and a 3-inch stroke.

Progesterone was used as a USP product of moderately fine subdivision. The same product was also ground in a Jet-O-Mizer, model 0202,[5] grinder. The progesterone thus ground had considerably finer particle size and an apparent density about one third that of the original material as judged by the volumes occupied by comparable weights. The progesterone was weighed into a 250-ml Erlenmeyer flask, wetted down with a 0.01 per cent aqueous Tween 80[6] solution, and shaken. The flask was then exposed to live steam at atmospheric pressure for 30 min. The progesterone

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[2] Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

[3] We wish to thank Dr. H. B. Woodruff for making this particular culture available to us.

[4] Sheffield Chemical Co., Norwich, New York. Mention of commercial brand names does not imply recommendation over any other similar type.

[5] Manufactured by Fluid Energy Processing and Equipment Company, Philadelphia, Pennsylvania.

[6] Atlas Powder Company, Wilmington, Delaware.

could not be autoclaved under the usual sterilizing conditions because it frequently melted and on cooling gave a product which would not disperse properly in the nutrient medium.

In the case of *R. nigricans*, 50 ml of an 18 to 24 hr vegetative culture was added to the flask containing the progesterone. With *A. ochraceus*, 25 ml of the vegetative culture was added along with 25 ml of sterile water to the flasks containing the progesterone. The flasks were shaken at 220 rpm on a 2-inch stroke rotary shaker for 24 hr or longer. In one experiment a larger scale run was conducted with *A. ochraceus* using 250 ml of the vegetative culture diluted with 250 ml of sterile water. In this case the fermentation vessel was a 4-L serum bottle aerated at a rate of 0.1 volume of air per volume of medium per min. The serum bottle was incubated on the rotary shaker described above.

For assay purposes the entire broth plus mycelium was extracted at least three times, using each time a volume of chloroform equal to the volume of the broth. The chloroform extracts were combined, filtered, and evaporated to dryness on the steam bath. The samples were analyzed by paper chromatogram techniques and, in some cases, by macro isolation procedures as well as the micro paper chromatogram. As shown in table 1 the two methods gave very good checks.

Paper chromatogram. The method is basically a modification of the typical Zaffaroni procedure (Zaffaroni *et al.*, 1950; and Zaffaroni and Burton, 1951). Whatman No. 4 paper was impregnated with 30 per cent propylene glycol in acetone. Aliquots of sample corresponding to approximately 200 μg were spotted along with known amounts of 11α-hydroxyprogesterone. The paper chromatogram was then developed with a 1:1 mixture of benzene and cyclohexane. The paper was removed when the solvent front was within 1 inch of the bottom (usually 1½ to 2 hr at 26 C). With the system used, unreacted progesterone moves just in back of the solvent front, 11α-hydroxyprogesterone moves 7 to 8 cm from the origin, and 6β, 11α-dihydroxyprogesterone does not move. The method is essentially a determination of 11α-hydroxyprogesterone. The paper sheets were thoroughly dried at 90 to 100 C and sprayed with vanillin-phosphoric acid spray (McAleer and Kozlowski, 1957).

Macro isolation. The dried chloroform extracts obtained as described above were dissolved in a minimal quantity of hot benzene. Heptane was added to the hot mixture until the first appearance of a cloud point. The solution was cooled to room temperature and the crystalline crop removed by filtration. The mother liquors were then heated on the steam bath to drive off more benzene until another cloud point was noted. The procedure was repeated several times. All crops melting above 150 C (usually after three crops a sharp fall in melting point denoting progesterone was observed) were combined, recrystallized from heptane-methanol, and weighed. The purity of the 11α-hydroxyprogesterone obtained in this manner was checked by paper chromatogram, melting point and infrared spectroscopy, and in all cases the preparations were found to be substantially pure. (In all cases paper chromatogram assay showed the absence of progesterone and 6β, 11α-dihydroxyprogesterone.)

Results and Discussion

The data for the 11α-hydroxylation of progesterone by *R. nigricans* and *A. ochraceus* are given in tables 1

TABLE 1

Conversion of progesterone to 11α-hydroxyprogesterone by Rhizopus nigricans

No.	Steroid Treatment	Progesterone*	Time	11α-Hydroxyprogesterone	
				Found†	Conversion‡
		g/L	hr	g/L	%
1	None	1.0	6	0.5	50
2	None	1.0	24	0.6	60
3	None	2.0	24	1.2	60
4	None	4.0	24	2.4	60
5	Ground	1.0	6	0.6	60
6	Ground	2.0	6	0.4	20
7	Ground	1.0	24	0.65	65
8	Ground	2.0	24	1.4	70
9	Ground	4.0	24	2.8	70
10	Ground	8.0	24	4.0	50

* Volume of all samples = 50.0 ml. Actual quantity of progesterone used is 1/20 amount shown.

† Paper chromatogram assay.

‡ Based on initial progesterone content.

TABLE 2

Conversion of progesterone to 11α-hydroxyprogesterone by Aspergillus ochraceus

No.	Steroid Treatment	Progesterone*	Time	11α-Hydroxyprogesterone	
				Found†	Conversion‡
		g/L	hr	g/L	%
11	Ground	20.0	24	14.0	70
12	Ground	20.0§	72	14.8	74
13	Ground	20.0	72	17.4	87
14	Ground	20.0¶	72	18.0	90
15	None	50.0	72	20.0	40
16	Ground	50.0	72	32.5	65
17	Ground	100.0	72	24.0	24

* All samples except that indicated by ¶ have volume of 50 ml and actual progesterone content is 1/20 that shown.

† Assay by direct isolation of crystalline 11α-hydroxyprogesterone. Paper chromatogram assay gave values in excellent agreement.

‡ Based on initial progesterone content.

§ Mycelium and nutrient media were not diluted 1:1 with water prior to addition to steroid.

¶ Actual volume is 500 ml; actual progesterone content is ½ that shown.

and 2, respectively. Our strain of *R. nigricans* under these experimental conditions was able to carry out 11α-hydroxylation of progesterone with reasonable efficiency up to concentrations of 8 g per L. At this point the hydroxylation rate began to decrease relative to that obtained with *A. ochraceus* (compare no. 10, table 1 with no. 11, table 2). Accordingly, only *A. ochraceus* was tested with the high concentrations of progesterone shown in table 2. The data shown in this latter table indicate that concentrations of 20 g of progesterone per L can be almost quantitatively converted to the 11α analogue and that even in concentrations of 50 g per L, 65 per cent conversion can be obtained.

There are several features of our experimental procedures which are noteworthy. The chief factor was the use of the Fluid Energy Mill[5] to grind the progesterone into very finely divided particles thus omitting the use of organic solvents. As a result, the problem of solvent toxicity in runs containing high concentration of starting materials was eliminated (*cf.* Karow and Petsiavas, 1956). The use of Tween 80 in low concentration resulted in a uniform wetting of the finely ground progesterone. Without the use of Tween 80 the finely ground particles floated on top of the medium in a talcum-like manner and prevented a uniform dispersion in the fermentation medium. The dilution of the growing culture was necessary for good agitation in fermentations using high concentrations of starting material, since at these concentrations the medium became very viscous.

Although the standard time period (72 hr) for the *A. ochraceus* experiments was considerably longer than the fermentation periods used by Dulaney *et al.* (1955) and Karow and Petsiavas (1956), it is of interest to note that very little of the 6β,11α-dihydroxyprogesterone was formed in the high concentration experiments. When similar fermentations were conducted at low concentrations, 0.5 to 1.0 g per L, we found extensive formation of the dihydroxyprogesterone. Hence one can conclude that so long as some progesterone remains in the fermentation medium, it will be preferentially hydroxylated to the mono-hydroxy form and that there will be little or no hydroxylation of the 11α-hydroxyprogesterone until all the unreacted progesterone has been mono-hydroxylated. For this reason dihydroxylation apparently occurs more readily in fermentations at low concentration.

Although in our experimental procedure we extracted both the mycelium and the fermentation medium, it has been found in later experiments that when high concentration of substrates is used the extraction of the fermentation medium is unnecessary. With substrate concentrations of 20 g per L, it was found that on filtering the mycelium 95 per cent of the total steroid was associated with the mycelium and could be extracted from the latter with acetone. Hence the usually awkward and laborious extraction of the aqueous phase can be omitted. This statement does not hold for fermentations at low concentration.

The procedure described in this paper for the fermentation of progesterone in high concentrations appears to be specific for this substrate. While studying the possible formation of 11α-hydroxy products from compound S, 16α,17α-epoxyprogesterone, and 16α,17α-epoxypregnane-3,20-dione, evidence was obtained that the 11α-hydroxy analogues may exert an inhibitory action on the course of the fermentation. Some data obtained with the latter steroids and with progesterone may explain why these hydroxylations are so specific. In these experiments mixtures of progesterone (P), 16α,17α-epoxypregnane-3,20-dione (EP), and their corresponding 11α-hydroxy analogues (11P and 11EP, respectively) were hydroxylated by *R. nigricans* under the experimental conditions previously described, except that rather low concentration levels were used with a small volume of propylene glycol as a solvent. The conversion of EP to 11EP has been previously described by Kenney *et al.* (1958). *A. ochraceus* was not used because this organism produces extensive degradation of EP and 11EP. The data are shown in table 3.

Steroids 11P and 11EP are easily separated by paper chromatography and hence mixtures of these could be

TABLE 3

Inhibition of the microbiological hydroxylation of progesterone and 16α,17α-epoxypregnane-3,20-dione in the presence of 16α,17α-epoxy-11α-hydroxypregnane-3,20-dione

No.	Steroid*	Conversion†
	mg/50 ml	%
18	20P	70 11P
19	20EP	30 11EP
20	20EP 20 11EP	0 11EP‡
21	20EP 20 11P	30 11EP
22	20P 20EP	75 11P; 11EP also present
23	20P 20 11P	80 11P‡
24	20P 20 11EP	30 11P
25	20P 10 11EP	55 11P
26	20P 5 11EP	75 11P

* Code P = progesterone, 11P = 11α-hydroxyprogesterone
EP = 16α,17α-epoxypregnane-3,20-dione
11EP = 16α,17α-epoxy-11α-hydroxypregnane-3,20-dione

† To 11α-hydroxy steroid in 24 hr.

‡ Allowance was made for the quantity of 11α-hydroxysteroid initially present.

estimated. The experiments clearly show that 11P does not inhibit the hydroxylation of P or of EP (*cf.* table 3, experiments 21 and 23, and compare with 18 and 19). Steroid EP also does not inhibit the formation of 11P from P (experiment 22). However 11EP has a marked inhibitory action on the hydroxylation of both its 11-deoxy-analogues, steroids EP and P, as shown in experiments 20 and 24. Moreover, as the quantity of 11EP was reduced, conversion of P to 11P was increased (experiments 24, 25, and 26). Our experiments do not permit conclusions as to the nature of the inhibition of the hydroxylation of P and EP by 11EP. It is apparent, however, that 11α-hydroxyprogesterone is noninhibitory to the hydroxylation of progesterone. The noninhibition of hydroxylation by large concentrations of 11P may well be exceptional.

Summary

Progesterone can be converted to 11α-hydroxyprogesterone by *Aspergillus ochraceus* in high yields at concentrations of 20 to 50 g per L. The fermentation is conducted with finely ground steroid in the absence of organic solvents using a very small concentration of wetting agent to provide dispersion of the solids in the fermentation medium. It is shown that 11α-hydroxyprogesterone does not inhibit the hydroxylation of progesterone but that 16α,17α-epoxy-11α-hydroxypregnane-3,20-dione is inhibitory toward hydroxylation of both progesterone and 16α,17α-epoxypregnane-3,20-dione.

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